Immuoassay of Digoxin by Differential Centrifugation


We describe an immunoassay of digoxin that exploits the differential sedimentation rate between two types of latex particles in a microcentrifugal analyzer. Conventional polystyrene latex particles (relative density = 1.05), sensitized with antibody, are first suspended with the sample and then mixed with digoxin-sensitized latex particles (relative density = 1.5). In the absence of digoxin in the sample, the two particle types bind to each other and, when subjected to centrifugal force, are cleared from the solution simultaneously. In the presence of digoxin, the binding between the particles is inhibited in proportion to the concentration of the drugs; the particles therefore can be differentially sedimented in 1 min. We measured the resulting absorbance to quantify the number of less-heavy particles and used the assay to generate a linear standard curve from 0 to 5 μg/L. Because the detection limit of the assay depends on the concentrations of both particle types, we optimized the assay by using two-dimensional analysis. Interference from rheumatoid factor was negligible up to 800 kilo-int. units/L. Precision studies indicated CVs between 5% and 13% within-run and between 4% and 9% for run-to-run in this endpoint assay.

Additional Keyphrases: latex assay  response surface analysis two-particle immunoassay  centrifugal analyzer

Traditional applications of centrifugal analyzers in clinical diagnostics have predominantly exploited their capacity to monitor multiple reactions essentially simultaneously (1). With a few exceptions (2, 3), the consequential presence of a centrifugal force has been largely overlooked or even considered detrimental (4) in most immunological applications. Outside of clinical diagnostics, however, sedimentation has been a useful analytical tool, e.g., in classical studies of particle sizing by sedimentation field flow fractionation techniques (5) and, for bioseparation processes in two-phase aqueous systems, in centrifugal partition chromatography (5). Only recently has a centrifugal force been used as an enhancement mechanism for immunoassays (2, 7).

Here we describe a technique whereby a centrifugal field is used to effect in situ separation of two latex particles that differ in their relative densities, and we apply this technique in an optimized immunoassay of digoxin. Application of this technique to assay of choriongonadotropin and some preliminary results for digoxin have been presented elsewhere (8). In a related two-particle method, a magnetic field was used to separate paramagnetic from nonparamagnetic particles (9). The great advantage of more speedy separation of particles by differential centrifugation, however, has allowed for a more detailed study of particle/particle stoichiometry in an immunoassay in which both the ligand and binder are in solid phase.

Materials and Methods

Materials

For the immobilization of antibody, we used unmodified polystyrene latex particles (about 0.8 μm in diameter), obtained as a 100 g/L suspension (Seragen, Indianapolis, IN). High-density latex particles (102 g/L) were obtained from Japanese Synthetic Rubber Co. America, New York, NY. Digoxin (USP grade XIX) was from Boehringer Mannheim, Indianapolis, IN. Whole rabbit serum containing anti-digoxin antibody was obtained from International Immunology Corp., Murrietta, CA. Pooled, delipidated whole human serum (Fisher Diagnostics, Orangeburg, NY) was tested to be digoxin-free by RIA (Organon Teknika Corp., Irving, TX). For interference studies, we used rheumatoid factor-containing pooled human serum (Bio-Plasma Inc., Voorhees, NJ), which was determined by the Fisher LAtest™-RF slide test to contain about 1600 int. units of rheumatoid factor per milliliter, and Fisher Diagnostics' Sera Chem Elevated Bilirubin Control Serum (lot no. 224-085), which was diluted with delipidated control serum. The suspension buffers used for the latex reagents contained (per liter): for the lighter particles, 66.5 mmol of (Na⁺–K⁺) phosphate (pH 7.2), 0.13 mol of NaCl, 1 g of bovine serum albumin (BSA), 1 mL of Tween 20 polyethylene sorbitan monolaureate, and 1 g of NaNO₃; for the heavy particles, 10 mmol of glycine–NaOH (pH 7.5), 0.15 mol of NaCl, 10 g of BSA, 1 mL of Tween 20, and 1 g of NaNO₃. All other materials were of reagent grade and obtained from Fisher Scientific Co., Fair Lawn, NJ.

Antibody-Sensitized Low-Density Latex Reagent

Dilute the 100 g/kg suspension of unmodified polystyrene latex particles (0.81 μm diameter, d = 1.05) as obtained from the manufacturer by approximately 170-fold in sodium phosphate buffer (50 mmol/L, pH 7.6), then pellet by centrifuging at 17 000 × g for 20 min. Resuspend the particles in the same phosphate buffer to give a concentration of 120 g/kg, as determined by measuring the turbidity of the suspension at 650 nm. Then add whole (rabbit) anti-digoxin antiserum (protein concentration about 70 mg/mL) directly to the latex suspension in a glass vial at a ratio of 200 μg of protein per milligram of latex, the final concentration of latex being 100 g/kg. Let the protein adsorb either overnight at 4°C or for 4 h at room temperature with continuous mixing by rolling the suspension on a hematologic mixer. Wash the particles three times by resuspending to an approximate concentration of 25 g/L in phosphate-buffered saline containing 1 g of BSA per liter. Finally, resuspend the particles to a working concentration of 3 g/L as determined by measuring absorbance at 650 nm in the same phosphate–BSA buffer.

Digoxin-Sensitized High-Density Latex Reagent

Protein–hapten synthesis. Prepare the protein–hapten conjugate of digoxin and BSA by oxidation with periodate, followed by reduction with sodium borohydride as described by Butler and Tse-Eng (10). Purify by gel filtration over
Sepharose G-100 or by "FPLC" (Mono Q column), both from Pharmacia. Typically, between 4 and 10 mol of digoxin are incorporated per mole of protein, as determined by the molar absorptivity of digoxin at 383 nm in 5 mol/L sulfuric acid.

Modification of the latex surface. Modify the high-density latex by covalent linkage with 1,6-hexanediamine. The rationale for modifying the latex surface by adding amine groups stems from the fact that the most of the sterically accessible linkage sites on the protein conjugate are occupied by the hapten in Schiff base linkage, leaving fewer of these sites to link through the carboxyl groups on the latex surface. The carboxyl groups on the protein should, however, remain free after covalent linkage with the latex.

First wash the latex (102 g/kg) by resuspending it in 0.1 mol/L sodium phosphate buffer (pH 6.2) and centrifuging, 500 × g, for 15 min. Resuspend the latex to a concentration of 10 g/kg in phosphate buffer in a glass graduated cylinder. Then add 1,6-hexanediamine (three times recrystallized in an equivolume solution of tetrahydrofuran/hexane), 0.6 g/kg of latex, to a final 1,6-hexanediamine concentration of 0.4 mol/L (100-fold molar excess of surface carboxyl groups).

Without delay, dilute the suspension to 0.1 mol/L with 1-ethyl(2-dimethylaminopropyl)carbodiimide (EDC) and incubate for 2.5 h at ambient temperature with gentle agitation. Wash the latex five times by repeated centrifugation in 0.01 mol/L phosphate buffer (pH 6.2) to remove all unreacted contaminants. The modified latex particles remain useful for weeks under refrigerated storage.

Covalent bonding of protein–hapten conjugate to latex. To the latex suspension that had been adjusted to a concentration of 100 g/kg in 0.01 mol/L phosphate buffer (pH 6.2) add solid EDC (final concentration 50 mmol/L). Immediately after the carbodiimide dissolves, add the protein–hapten conjugate at a ratio of 30 μg of conjugate protein per milligram of latex particles and agitate the reaction mixtures gently by turning the vials end over end at room temperature overnight. Wash the latex particles free of unreacted materials by first centrifuging then resuspending them in Tris HCl buffer (15 mmol/L, pH 7.0) containing 30 mL of Triton X-100 and 0.5 mol of NaCl per liter. After incubating at room temperature for 2 h with gentle agitation, centrifuge (pellet) the latex and repeat the procedure. We generally perform between five and eight washing cycles. After a final wash, resuspend the latex in glycine–NaOH buffer.

Assay Procedure

We wrote a program for use with the Multistat III Plus centrifugal analyzer (Instrumentation Laboratory, Lexington, MA), which makes use of a PDP-8 computer, to adapt to the differential centrifugation immunoprocess. Essentially, we structured the program using focal™ (Digital Equipment Corp.) programming language into several subroutines. After pre-incubating the reagents undisturbed in the rotor, the instrument (a) rapidly accelerates the rotor to mix the reagents, and (b) enters a 20-s mixing cycle in which the rotor is halted and restarted every second. To differentially sediment the particles in 1 min, we alternate between "run" (80 × g) and "accelerate" (320 × g) modes such that the average centrifugal force is approximately 200 × g. The rotor then slows for data acquisition and prints out the absorbance at 380 nm of samples vs water in the reference cuvette. For convenience, we use the average absorbance of the first two sample cuvettes, which contain the serum blanks, and subtract this from the absorbance of the subsequent cuvettes.

Typically, the assay is performed as follows. We placed the conventional polystyrene latex particles (d = 1.05) with adsorbed antibody in the sample chamber of the Multistat rotor, along with the serum sample to be analyzed. The analyzer mixes the suspension with latex particles of a higher relative density (d = 1.50), which have been sensitized with the corresponding antigen (i.e., digoxin). For these experiments, we loaded the reagents into the rotor by the Multistat Model 770 loader (Instrumentation Laboratory). Each sample chamber thus contained 20 μL of human serum sample with or without digoxin, 5 μL of a 3 g/kg suspension of antibody-sensitized latex (0.81-μm-diameter particles suspended in phosphate-buffered saline containing BSA, 10 g/L), and 25 μL of water (diluent). The corresponding reagent chamber contained 25 μL of a 50 g/kg suspension of the high-density latex particles (sensitized with digoxin) in glycine buffer. As diluent, 70 μL of water was added, to make a total volume of 145 μL after mixing.

Results

Determination of Nonspecific Binding

After determining, in control experiments, the optimal rotor speed and centrifugation time needed to separate the light and heavy particles, we could quantitatively assess the antibody-mediated particle–particle binding. The percent of binding, defined as that percentage of the total amount of the low-density latex reagent removed from the reaction mixture after pelleting all of the high-density latex, was determined from the absorbance of the suspension at 380 nm. In the absence of analyte, we titrated a fixed amount of low-density latex reagent with high-density latex, which typically precipitated between 60% and 80% of the antibody-sensitized lighter particles when present at or near saturating conditions, i.e., latex concentration, 20 g/L (8). Depending on various factors involved in the synthesis of the two particulate reagents, we could obtain as much as 95% binding. When a similar experiment was performed with lighter particles adsorbed with nonimmune (pre-immunization) rabbit serum, only 2.5% of the total latex added was precipitated by digoxin-sensitized high-density latex, even at final concentrations as great as 17 g/L. Similarly, when BSA alone was immobilized on the surface of the high-density latex and the anti-digoxin antibody on the lighter particles, very little (<1%) of the added low-density latex was bound. Investigation of the pH dependence of binding demonstrated an optimum between pH 7.0 and 8.0.

Appropriate conditions for establishing a standard curve by our two-particle method should be such that the binding between the particles can be inhibited by a clinically significant concentration of analyte. Classical optimization of immunoprocesses in which binder and ligand are both soluble are based on this criterion (11). When we added digoxin (0.5 to 8.0 μg/L) to drug-free normal human serum, we could attain the sensitivity required for determining therapeutic concentrations of digoxin, but the standard curve exhibited a hyperbolic shape (8), which may be indicative of suboptimal assay conditions at higher concentrations of the hapten (>2 μg/L). For simplicity in the use of curve-fitting routines, the dose–response relationship should be linear throughout the working range. In addition, a linear relationship yields simpler and uniform error analyses. Thus we decided to optimize the assay further.
Assay Optimization

Several studies of antibody–hapten binding kinetics have described the much shorter time required to form an immune complex than to dissociate it (12). Guided by this fundamental principle, we used the sequential saturation principle (13) to optimize our two-particle assay, first allowing the sample ligand to reach equilibrium with the antibody before filling the remaining binding sites with an excess of the labeled ligand.

Before mixing with the high-density reagent, we determined the time required for the free analyte to reach equilibrium with the antibody-sensitized lighter particles. After loading the digoxin-containing standards and the lighter particles into the sample chamber of the Multistat rotor, then loading the high-density latex into the reagent chamber, we incubated the rotor for various periods before initiating the reaction. From the family of standard curves shown in Figure 1, we determined that steady state was approached after about 5 min of incubation at 30 °C. Therefore, to ensure that equilibrium between the free analyte and the antibody immobilized on the lighter particles was always reached, we used a 5-min pre-incubation before mixing the two latex reagents for our remaining experiments.

To determine the relative effect of the concentrations of the two latex particles on the extent of binding, we performed a two-dimensional titration, varying the concentration of the low-density latex reagent from 0.05 to 0.50 g/kg and that of the high-density latex reagent from 1.2 to 20.8 g/kg in the absence of analyte. Figure 2 illustrates the results of this experiment, expressed as the percentage of the total low-density latex added that was bound and cleared from suspension by the high-density latex. Although one might expect that a low concentration of the antibody-bearing low-density particles would be saturated with proportionately fewer heavy particles, in analogy to a system in which one or both of the reagents are soluble (11), we found that each concentration of lighter particles was saturated at nearly the same concentration of high-density latex, about 4.0 g/kg. Because both molecular reactants are immobilized on particles, the frequency of collisions between the particles plays a major role in their reactivity. In a system in which all of the components are soluble, the rate of molecular diffusion is usually the limiting factor. In a solid-phase assay such as we have described, however, the frequency of particle–particle collisions should depend on the total concentration of particles in the reaction. The total particle concentration in the vicinity of saturation in our two-dimensional titration varies only slightly (8.24 × 10^6 to 10.6 × 10^6 particles/mL) over the range studied, by less than a factor of 1.3. From these data we found that the efficiency with which the particles bound to each other was largely a function of the total particle concentration. However, we did not find this to be entirely the case during the optimization of assay sensitivity.

The ability of free hapten to compete for the available antibody with the immobilized hapten on the heavier particles might also be affected by steric factors, in light of the results described above. The affinity of the high-density latex reagent for antibody in our assay system may be influenced by: (a) the number of hapten molecules on the protein conjugate itself, (b) immobilization of the conjugate on a solid support, and (c) the possibility that the antibody recognizes the bridge group, not just the analyte (14). Therefore, to more rigorously optimize the two-particle system with respect to both particle types, we constructed a two-dimensional plot to create a response surface reflecting the behavior of the analytical configuration in the presence of analyte (2 ng/mL).

We used a 10 × 18 matrix of differing concentrations of each particle type and plotted the results as the difference in absorbance between the experimental system and the control, which contained no digoxin (Figure 3). The number of antibody sites present (on the lower-density particles) dramatically affected the sensitivity (detection limit) typical for these immunoassays. An apparently well-defined optimal ridge was generated when the final concentration of the lower-density latex particles was 0.16 g/kg. This result is in contrast to the relatively negligible effect on the binding
described the digoxin high-density response approaching increased concentration curve particles density phenomenon by the identical concentration range of low-density particles (Figure 2). The concentration of heavy particles also affected sensitivity as indicated by the changing slope along the crest of the optimal ridge, although the curve never reached a point of inflection with respect to the concentration of the high-density latex. The signal intensity increased dramatically at high-density latex concentrations approaching 1.2 g/kg, the lowest concentration tested.

Using the conditions associated with the point on the response surface that resulted in the greatest signal, that is, high-density latex at 1.2 g/kg and low-density latex at 0.16 g/kg, we generated a standard curve for the immunoassay of digoxin (Figure 4). This curve displayed a linear response in the therapeutic range as compared to the hyperbolic curve described previously (8).

Assay Precision

Table 1 summarizes the precision data for the assay. The greater variation at lower digoxin concentrations (0.5 ng/mL) is probably due to the nature of an endpoint assay. Significant absolute cuvette-to-cuvette variations in the MCA rotor may also contribute to the high CV at low digoxin concentrations.

Interfering Substances

In an endpoint assay, background subtraction compensates only for those components in normal serum that absorb at 380 nm. At the assay conditions we described, digoxin-free serum containing 40 mg of bilirubin per liter resulted in an apparent digoxin concentration of 0.5 ng/mL. Concentrations of bilirubin >1.5 mg/L are considered abnormal for adults. The effects of hemolyzed or lipemic samples might also present problems in this regard, and are currently under investigation.

We also considered the effect of the presence of rheumatoid factor, known to be a potentially interfering substance in latex particle assays. As Table 2 shows, however, results of the differential centrifugation immunoassay were not affected by rheumatoid factor in the serum, at concentrations as great as 800 kilo-int. units/L.

Effects of Particle Size and Antibody Coating

To investigate the effect of particle size on the assay, we adsorbed 0.10-, 0.42-, 0.81-, and 1.05-μm-diameter polystyrene latex particles (d = 1.05) with the same rabbit anti-digoxin whole serum, such that the ratio of the total protein to the total surface area remained constant. Each of the particle preparations was then suspended at 0.20 g/kg and titrated with the same high-density latex reagent. To compare the four preparations, we used the amount of high-density latex reagent that would bind approximately 60% of the total lower-density anti-digoxin reagent. When we tested the response of each to a 3.0 ng/mL digoxin standard in human serum, we obtained the following order of decreasing response: 1.0 μm = 0.81 μm > 0.42 μm > 0.10 μm. The relative response characteristics of the various particle sizes may be influenced by a variety of factors, e.g., the wavelength used, the ratio of the surface areas of the lower-density to the heavier-density particles, and the total number of particles in the system. We chose to use the 0.81-μm-diameter particles.

To determine the effect of antibody coating on assay

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<th>Table 1. Precision of the Differential Immunoassay for Digoxin</th>
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<td>Digoxin concn in serum, μg/L</td>
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sensitivity, we adsorbed 0.81-μm-diameter low-density latex particles with different amounts of rabbit anti-digoxin serum, from 50 to 300 μg of protein per milligram of latex particles. We then saturated the particles with a nonselective protein, bovine serum albumin. As reported previously (8), the signal obtained for samples containing digoxin at 2.0 μg/L was roughly proportional to the concentration of anti-serum protein used, up to 200 μg/mL; greater protein concentrations did not further increase the absorbance.

Discussion

Several other two-particle solid-phase immunoassay systems have been described, either in published literature or in patents. In one method, the particles are separated on the basis of their paramagnetic characteristics (9), while in the others, the second particle is used to enhance agglutination (15, 16). The dynamics of the particle–particle interaction has not been documented for any of these systems. Here we describe in detail the functional relationships for an immunoassay in which both the ligand and binder are immobilized in solid phase. Unlike the previously described latex-bead assay (7), our method does not necessarily depend on agglutination, but can be described as "pseudo-heterogeneous," in that bound and free analyte are separated in situ. This separation approach may account for the potentially greater sensitivity achieved.

We first approximated the sensitivity dependence of the assay on the particle concentrations by using a factorial design experiment. Given the speed of the assay, optimization may be most efficiently accomplished by the sequential simplex method (17); for investigational purposes, however, a complete surface profile was more informative.

Under the described protocol, soluble analyte is in contact with antibody until steady-state is reached and the maximum number of binding sites is occupied. In keeping with the sequential saturation method (13), we added high-density reagent in excess of the remaining binding sites, allowing for a shorter incubation time. In addition to functioning as a separating agent, however, the concentration of antigen-bearing particles also affects assay sensitivity. Superimposition of the three-dimensional titration and sensitivity plots reveals that the slope of the optimal sensitivity ridge begins to increase sharply at about the saturation point of the binding plot, analogous to the findings of a two-dimensional titration.

One disadvantage of an endpoint analysis such as this is the potential for a high background absorbance from substances that absorb or scatter light at a wavelength similar to the wavelength of measurement (18). Among the suggestions to overcome this potential problem are bichromatic blanking, dilution, and the use of a serum blank (19). Unfortunately, given the nonselective light-scattering properties of latex particles, it is not feasible to find two wavelengths suitable for use in bichromatic blanking. Secondly, dilution would be an effective alternative if sensitivity were not an issue: in the present study, we typically used only a ninefold final dilution of sample in the reaction cuvette, which means that the background contribution from the serum sample to the total absorbance reading could be significant. Another possibility would involve measuring the background absorbance after clearing the remaining low-density particles from the reaction mixture by re-initiating a centrifugation cycle. This background absorbance could be subtracted from the absorbance of the first data acquisition (after removal of the high-density particles) to obtain a corrected absorbance, but would require additional assay time (15 min at 200 × g).

A more appealing possibility for overcoming interference is to immobilize an enzyme as well as the antibody on the lighter particle. In this case the high-density reagent would be suspended in substrate solution and the lighter particles remaining in suspension after sedimentation of the heavier latex would catalyze the formation of a color or fluorescence. The enzyme on the lighter particles that bound to the pelleted high-density reagent would be sequestered from the soluble substrate. This setup would allow for kinetic rate measurements.

Some of the advantages of the differential centrifugation immunoassay over conventional latex agglutination include greater sensitivity, speed, and relatively low interference from serum proteins. Some of these advantages can be attributed to the consideration that only a single binding event rather than the formation of multimeric aggregates is theoretically necessary to detect a response, although the formation of multimers during centrifugation cannot be ruled out.

The detection limit of this two-particle method for digoxin exceeds that claimed for most other latex assays and certainly challenges the enzyme-mediated immunoassays. When we used this configuration to assay human choriongonadotropin, the detection limit was 8 int. units/L (8). Although these studies were performed with a completely automated centrifugal analyzer, the assay can be easily formatted to a manual, single-dose configuration for a desktop centrifuge.

References